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(J-1500)

TITLE

Compositions and Methods for Detection of Antibody Binding to Cells

CROSS REFERENCE TO RELATED APPLICATIONS

This application ~~claims~~ priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 60/028,550, filed on October 11, 1996.

GOVERNMENT SUPPORT

This invention was supported in part by a grant from the U.S. Government (NIH Grant No. P50-HL54516) and the U.S. Government may therefore have certain rights in the invention.

FIELD OF THE INVENTION

The field of the invention is agglutination of cells.

BACKGROUND OF THE INVENTION

Each year, tens of millions of units of blood are collected worldwide and an equal number of patients receive those units of blood as transfusions. Every unit of collected blood and every patient must be typed for the Rh antigen to ensure a match prior to the transfusion. The ID-Micro Typing System, Inc., disclosed in U.S. Patent No. 5,338,689 (Ortho Diagnostics, Inc.) provides a simplified method for antigen typing of red blood cells. Other micro typing card systems are also knaown in the art. For example, a DiaMed AG card system is available from Cressier, Switzerland, known as the DiaMed-ID Micro Typing System. In addition, a Bio Vue card is available from Ortho Diagnostics.

In a conventional blood typing test, drops of typing serum and donor or recipient red blood cells are placed in test tubes and are incubated together. Excess

unreacted serum is washed away and a drop of rabbit anti-IgG antibody (Coomb's reagent) is added to the mixture to induce agglutination between cells that may have bound the typing reagent. This test is known as an indirect agglutination test or an indirect Coomb's test. Agglutination is assessed by briefly centrifuging the cells and gently shaking the tubes one by one over a concave mirror and observing the presence of red blood cell agglutinates as the cells return to a suspension. Microwell arrays in microplates may be used in place of test tubes.

In the Micro Typing System, red blood cells are centrifuged in a controlled manner through a dextran-acrylamide gel and Coomb's reagent predispensed in a specially designed microtube. Measured volumes of serum or plasma and/or red blood cells are dispensed into the reaction chamber of the microtube. If necessary, the card is incubated and then centrifuged. Agglutinated red blood cells become trapped in or above the gel and unagglutinated red blood cells travel through the gel particles and form a pellet at the bottom of the microtube.

In a second type of blood group detection system described in WO 9531731 A, a method of detecting a blood group antigen is disclosed. The method comprises adding a sample of red blood cells to a reaction tube which has a lengthwise axis containing a reaction medium consisting of several particles which have immunoglobulin-binding ligands selected from protein A, protein G, protein A/G or a universal kappa light chain binding protein, which ligands are coupled to the surface of the particles, and antibody, optionally a bridging antibody, specific for the antigen coupled to the ligand on the particles. The reaction tube is centrifuged for a time which is sufficient to remove red blood cells which have not attached to the antibody in the form of a pellet in the bottom of the tube. The attachment of the red blood cells, or the lack of attachment of red blood cells is detected and the attachment is correlated with the presence of the antigen.

Each of these methods is designed to detect red blood cell antigens using antibodies which have been produced in eukaryotic cells, either as monoclonal or

polyclonal antibodies. These methods cannot be used to detect antibodies which are expressed on the surface of virus particles.

The ability to produce monoclonal antibodies has revolutionized diagnostic and therapeutic medicine. Monoclonal antibodies are typically produced by immortalization of antibody-producing mouse lymphocytes thus ensuring an endless supply of cells which produce mouse antibodies. However, for many human applications, it is desirable to produce human antibodies. For example, it is preferable that antibodies which are administered to humans for either diagnostic or therapeutic purposes are human antibodies since administration of human antibodies to a human circumvents potential immune reactions to the administered antibody, which reactions may negate the purpose for which the antibody was administered.

In addition, there exist certain situations where, for diagnostic purposes, it is essential that human antibodies be used because other animals are unable to make antibodies against the antigen to be detected in the diagnostic method. For example, in order to determine the Rh phenotype of human red blood cells, human sera that contains anti-Rh antibody must be used since no other animal can make an antibody capable of detecting the human Rh antigen.

The production of human antibodies *in vitro* by immortalizing human B lymphocytes using Epstein Barr virus (EBV)-mediated transformation or cell fusion has been fraught with technical difficulties due to the relatively low efficiency of both EBV-induced transformation and cell fusion when compared with the murine system. To overcome these problems, processes have been developed for the production of human antibodies using M13 bacteriophage display (Burton *et al.*, 1994, *Adv. Immunol.* 57:191-280). Essentially, a cDNA library is generated from mRNA obtained from a population of antibody-producing cells. The mRNA encodes rearranged immunoglobulin (Ig) genes and thus, the cDNA encodes the same. Amplified cDNA is cloned into M13 expression vectors creating a library of phage which express human Fab fragments on their surface. Phage which display the antibody of interest are selected by antigen binding and are propagated in bacteria to produce soluble human

Fab Ig. Thus, in contrast to conventional monoclonal antibody synthesis, this procedure immortalizes DNA encoding human Ig rather than cells which express human Ig.

There are several difficulties associated with the generation of antibodies using bacteriophage. For example, many proteins cannot be purified in a non-denatured state, in that purification procedures necessarily involve solubilization of protein which may render some proteins permanently denatured with concomitant destruction of antigenic sites present thereon. Such proteins thus cannot be bound to a solid phase and therefore cannot be used to pan for phage bearing antibodies which bind to them. An example of such a protein is the human Rh antigen.

To solve the problem, a method was developed wherein intact red blood cells were used as the panning antigen (Siegel *et al.*, 1994, *Blood* **83**:2334-2344). However, it was discovered that since phage are inherently "sticky" and red blood cells express a multitude of antigens on the cell surface, a sufficient amount of phage which do not express the appropriate antibody on the surface also adhere to the red blood cells, thus rendering the method impractical for isolation of phage which express antibody of desired specificity.

De Kruif *et al.* (1995, *Proc. Natl. Acad. Sci. USA* **92**:3938-3942) disclose a method of isolating phage encoding antibodies, wherein antibody-expressing phage are incubated with a mixture of antigen-expressing cells and cells which do not express antigen. The antibody-expressing phage bind to the antigen-expressing cells. Following binding with phage, a fluorescently labeled antibody is added specifically to the antigen-expressing cells, which cells are removed from the mixture having antibody-expressing phage bound thereto. The isolation of fluorescently labeled cells is accomplished using the technique of fluorescently-activated cell sorting (FACS), an expensive and time-consuming procedure.

There is a need for a method of isolating recombinant proteins, preferably antibodies, which is rapid and economical, and which will provide a vast

array of protein-binding proteins useful for diagnostic and therapeutic applications in humans.

There is also a need for rapid and accurate assays for the typing of red blood cells using recombinant proteins which are expressed on a virus surface. The present invention satisfies these needs.

SUMMARY OF THE INVENTION

The invention relates to a method of agglutinating cells comprising providing a mixture comprising a population of cells and a population of bacteriophage expressing a first antibody on the surface of the bacteriophage, the first antibody being specific for an antigen-bearing moiety expressed by at least a portion of the cells in the cell population, wherein the first antibody binds to the portion of the cells causing the bacteriophage to also bind to the portion of the cells, adding to the mixture a second antibody specific for the bacteriophage, wherein binding of the second antibody to bacteriophage bound to the portion of the cells causes the portion of the cells to agglutinate.

Also included in the invention is a method of detecting cell agglutination, comprising providing a mixture comprising a population of cells and a population of bacteriophage expressing a first antibody on the surface of the bacteriophage, the first antibody being specific for an antigen expressed by at least a portion of the cells in the cell population, wherein the first antibody binds to the portion of the cells causing the bacteriophage to also bind to the portion of the cells, adding the mixture to a microtube containing inert particles and a second antibody specific for the bacteriophage, allowing the mixture to sediment under the force of gravity, and observing the location of the portion of the cells, wherein strong agglutination of the portion of the cells is indicated by the cells being located upon or within a top layer of the inert particles and weak agglutination of the cells is indicated by the cells being located within a lower layer of the inert particles and no agglutination is indicated by the cells being located at the bottom of the microtube.

In one aspect, the step of sedimentation is effected by centrifugation.

The invention further relates to a method of capturing cells comprising providing a mixture comprising a population of cells and a population of bacteriophage expressing a first antibody on the surface of the bacteriophage, the first antibody being specific for an antigen expressed by at least a portion of the cells in the cell population, wherein the first antibody binds to the portion of the cells causing the bacteriophage to also bind to the portion of the cells, adding the mixture to a microtube containing inert particles which have bound thereto a second antibody specific for the bacteriophage, allowing the mixture to sediment under force of gravity, wherein captured cells are located upon or within a top layer of the inert particles.

In one aspect, the sedimentation step is effected by centrifugation.

In addition, the invention relates to a method of detecting capturing of cells comprising providing a mixture comprising a population of cells and a population of bacteriophage expressing a first antibody on the surface of the bacteriophage, the first antibody being specific for an antigen expressed by at least a portion of the cells in the cell population, wherein the first antibody binds to the portion of the cells causing the bacteriophage to also bind to the portion of the cells, adding the mixture to a microtube containing inert particles which have bound thereto a second antibody specific for the bacteriophage, allowing the mixture to sediment under force of gravity, and observing the location of the portion of the cells, wherein capturing of the portion of the cells is indicated by the cells being located upon or within a top layer of the gel particles and the absence of capturing of the cells is indicated by the cells being located at the bottom of the microtube.

In this method of the invention, the sedimentation step may also be effected by centrifugation.

The invention also relates to a method of detecting the presence of an antigen-bearing moiety on a cell comprising providing a mixture comprising a population of cells and a population of bacteriophage expressing a known first antibody on the surface of the bacteriophage, wherein the presence of the antigen-bearing moiety

on the cells is indicated by binding of the first antibody to at least two of the cells causing the bacteriophage to also bind to the at least two of the cells, wherein when a second antibody is added to the mixture which is specific for the bacteriophage the second antibody binds to bacteriophage bound to the at least two of the cells causing the cells to agglutinate, the agglutination being an indication of the presence of the antigen-bearing moiety on the cell, which antigen-bearing moiety is specific for the first antibody.

A method of identifying an antigen-bearing moiety on a cell is also included in the invention. This method comprises providing a mixture comprising a population of cells and a population of bacteriophage expressing a known first antibody on the surface of the bacteriophage, wherein the presence of the antigen-bearing moiety on the cells is indicated by binding of the first antibody to at least two of the cells causing the bacteriophage to also bind to the at least two of the cells, wherein when a second antibody is added to the mixture which is specific for the bacteriophage the second antibody binds to bacteriophage bound to the at least two of the cells causing the cells to agglutinate, wherein the agglutination identifies the antigen-bearing moiety as being an antigen-bearing moiety specific for the first antibody.

The invention further relates to a method of detecting the presence of an antigen-bearing moiety on a cell comprising providing a mixture comprising a population of cells and a population of bacteriophage expressing a known first antibody on the surface of the bacteriophage, wherein the presence of the antigen-bearing moiety on the cell is indicated by binding of the first antibody to at least two of the cells causing the bacteriophage to also bind to the at least two of the cells, adding the mixture to a microtube containing inert particles and a second antibody specific for the bacteriophage, allowing the mixture to sediment under the force of gravity, and observing the location of cell in the microtube, wherein strong agglutination of the cells is indicated by the cells being located upon or within a top layer of the inert particles which strong agglutination is an indication of the presence of the antigen-bearing moiety on the cell, which antigen-bearing moiety is specific for the first antibody.

The invention also includes a method of identifying an antigen-bearing moiety on a cell comprising providing a mixture comprising a population of cells and a population of bacteriophage expressing a known first antibody on the surface of the bacteriophage, wherein the presence of the antigen-bearing moiety on the cell is indicated by binding of the first antibody to at least two of the cells causing the bacteriophage to also bind to the at least two of the cells, adding the mixture to a microtube containing inert particles and a second antibody specific for the bacteriophage, allowing the mixture to sediment under the force of gravity, and observing the location of cells in the microtube, wherein strong agglutination of cells is indicated by the cells being located upon or within a top layer of the inert particles which strong agglutination identifies the antigen-bearing moiety as being an antigen-bearing moiety specific for the first antibody.

Also provided is a method of detecting the presence of an antigen-bearing moiety on a cell comprising providing a mixture comprising a population of cells and a population of bacteriophage expressing a known first antibody on the surface of the bacteriophage, wherein the presence of the antigen-bearing moiety on the cell is indicated by binding of the first antibody to at least two of the cells causing the bacteriophage to also bind to the at least two of the cells, adding the mixture to a microtube containing inert particles which have bound thereto a second antibody specific for the bacteriophage, allowing the mixture to sediment under force of gravity, wherein captured cells are located upon or within a top layer of the inert particles, the presence of the captured cells being an indication of the presence of an antigen-bearing moiety on the cell, which antigen-bearing moiety is specific for the first antibody.

In addition, the invention includes a method of identifying an antigen-bearing moiety on a cell comprising providing a mixture comprising a population of cells and a population of bacteriophage expressing a known first antibody on the surface of the bacteriophage, wherein the presence of the antigen-bearing moiety on the cell is indicated by binding of the first antibody to at least two of the cells causing the bacteriophage to also bind to the at least two of the cells, adding the mixture to a

microtube containing inert particles which have bound thereto a second antibody specific for the bacteriophage, allowing the mixture to sediment under force of gravity, wherein captured cells are located upon or within a top layer of the inert particles, the presence of the captured cells identifying the antigen-bearing moiety on the cell as being specific for the first antibody.

In one aspect of the methods of the invention, the cells are selected from the group consisting of red blood cells and white blood cells. Preferably, the cells are red blood cells.

In another aspect, the bacteriophage is M13 and the second antibody is anti-M13 antibody.

In yet another aspect, the first antibody is an anti-red blood cell antibody, preferably, an anti-Rh antibody.

In a further aspect, the antigen-bearing moiety is a red blood cell antigen or a HLA antigen.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram of a strategy for cell-surface Fab-phage panning using magnetically-activated cell sorting.

Figure 2 is a graph depicting cell-surface biotinylation of human red blood cells.

Figure 3 is a series of graphs which validate the antigen-positive, antigen-negative cell separation procedure of the invention.

Figure 4 is an image of a microplate agglutination assay wherein anti-Rh(D) Fab/phage agglutination titer was measured.

Figure 5 is an image of a microplate agglutination assay showing determination of Rh(D) binding epitope for selected anti-Rh(D) Fab/phage clones.

Figure 6 is an image depicting the use of Fab/phage antibodies in a gel card assay.

DETAILED DESCRIPTION

According to the present invention, there are provided rapid methods of typing cells with respect to the antigens expressed thereon, which methods are based upon the use of antibodies which are expressed on the surface of a virus. The typing of cells using phage-expressing antibodies is based on the use of an anti-phage antibody in an agglutination reaction.

Typically, the cells to be typed are red blood cells although as discussed herein, the invention is not limited solely to typing of red blood cells.

There are significant advantages to typing cells using a phage system, particularly in the case of typing red blood cells. For example, approximately 10% of blood donors and recipients have red blood cells which are coated with some of their own IgG antibodies. While the mechanism by which this occurs is immaterial, the presence of these antibodies on the red blood cells renders the cells untypeable by conventional methods which use IgG anti-Rh typing serum. For example, in a conventional assay, IgG anti-Rh typing serum is added to red blood cells . The unbound typing serum is washed, and Coombs reagent is added to induce agglutination. Coombs reagent is rabbit anti-human IgG. Red blood cells which are already coated with IgG will agglutinate whether or not the anti-Rh serum bound to them, that is, agglutination will occur whether the cells are Rh-positive or Rh-negative. This presents a dilemma because it is not possible to determine the subject's Rh phenotype. However, when phage antibodies are used in the assay, the agglutinating antibody (the bridging antibody) is anti-phage antibody. Thus, it becomes irrelevant to results of the assay that the cells are already coated with IgG. Thus, typing of red blood cells using phage antibodies permits typing of cells which have a false positive direct Coombs test result.

Another advantage of using a phage typing system is found in the superior sensitivity of the phage typing system over conventional systems. The standard indirect Coombs test requires approximately 150 to 3000 IgG molecules per red blood cell in order to yield a positive result. When anti-Rh(D)-phage antibody was

used in the assays described herein, only about 10-20 antibodies were required for a positive result. While not wishing to be bound to any particular theory, this approximate 10 to 100-fold increase in sensitivity is likely due to the long length (approximately 0.5 microns) of the phage which provides increased surface of the secondary antibody (i.e., anti-phage antibody) to bind and crosslink the cells.

Methods of producing antibodies which are expressed on the surface of a virus are first described herein, followed by a description of methods of typing red blood cells using virus expressed antibodies and anti-phage antibodies.

A novel method has been discovered for the isolation of DNA encoding a protein and the protein encoded thereby, wherein the protein is preferably an antibody, which protein is capable of specifically binding to an antigen-bearing moiety such as protein, a lipid, a carbohydrate, a nucleic acid and a complex of at least one of a protein, a lipid, a carbohydrate and a nucleic acid. The antigen-bearing moiety may be a membrane bound protein which is selected from the group consisting of an antigen and a receptor. In another aspect, the membrane bound protein is an antigen, such as a red blood cell antigen, such as Rh antigen. When the antigen-bearing moiety is a carbohydrate, it may be a carbohydrate expressed on a glycolipid, for example, a P blood group antigen or other antigen.

As exemplified herein but not limited thereto, the method comprises generating bacteriophage which encode human antibodies. Specifically, anti-Rh(D) red blood cell Fab/phage antibodies encoded by an M13 filamentous phage library are obtained. The library is generated from antibody-producing cells obtained from a hyperimmunized donor by first obtaining cDNA derived from mRNA expressed in the antibody-producing cells. Ig encoding fragments of the cDNA are obtained using the polymerase chain reaction (PCR) and primers specific for such fragments of DNA. Ig-specific DNA so obtained is cloned into a bacteriophage. Bacteriophage encoding the Ig fragments are panned against a mixture of antigen-positive, biotinylated red blood cell target cells pre-coated with streptavidin-conjugated magnetic microbeads and excess unlabeled red blood cells. Bacteriophage which express antibodies on the phage

surface, which antibodies are specific for the target cell antigen, bind to the labeled cells. These phage are separated from phage which are bound to unlabeled cells and from phage which are not bound to the cells using a magnetic column. Phage so separated encode and display antibody specific for antigens on the target cells.

To generate a phage antibody library, a cDNA library is first obtained from mRNA is isolated from cells which express the desired protein to be expressed on the phage surface, *e.g.*, the desired antibody. cDNA copies of the mRNA are produced using reverse transcriptase. cDNA which specifies Ig fragments are obtained by PCR and the resulting DNA is cloned into a suitable bacteriophage vector to generate a bacteriophage DNA library comprising DNA specifying Ig genes. The procedures for making a bacteriophage library comprising heterologous DNA are well known in the art and are described, for example, in Sambrook *et al.* (1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY).

A bacteriophage library may also be obtained using cDNA rather than PCR-amplified Ig encoding fragments of cDNA. Generation of a cDNA library is useful for isolation of proteins which are not antibodies, such as ligands and the like.

Bacteriophage which encode the desired protein, *e.g.*, an antibody, may be engineered such that the protein is displayed on the surface thereof in such a manner that it is available for binding to its corresponding binding protein, *e.g.*, the antigen against which the antibody is directed. Thus, when bacteriophage which express a specific antibody are incubated in the presence of a cell which expresses the corresponding antigen, the bacteriophage will bind to the cell. Bacteriophage which do not express the antibody will not bind to the cell.

For panning of bacteriophage, *i.e.*, selection of phage which express the desired antibody, cells which express the corresponding antigen are labeled with a detectable label such as biotin. Streptavidin-conjugated magnetic beads are then added to the cells. The cells are mixed with an excess of unlabeled cells which do not express the antigen. This cell mixture is then incubated with the phage library, wherein phage which express the antibody bind to cells expressing the antigen. The presence of the

excess unlabeled cells in the mixture serves as a means of removing bacteriophage which do not express the antibody but which might otherwise bind to antigen-expressing cells non-specifically. The details of the experimental procedures are provided herein in the experimental detail section.

Antigen-expressing cells having antibody-expressing phage bound thereto are magnetically removed from the mixture. One example of magnetic removal involves pouring the mixture of magnetic and non-magnetic cells into a column in the selective presence or absence of a magnetic field surrounding the column. Alternatively, magnetic cells may be separated from non-magnetic cells in solution by simply holding a magnet against the side of a test tube and attracting the cells to the inner wall and then carefully removing the non-magnetic cells from the solution.

Thus, the method just described involves a procedure for enriching a population of recombinant phage for those expressing specific phage-displayed ligands derived from natural or synthetic phage DNA libraries by simultaneously performing negative and positive selection against a mixture of magnetically-labeled receptor-positive particles (*i.e.*, cells) and unlabeled receptor-negative particles.

The terms "bacteriophage" and "phage" are used interchangeably herein and refer to viruses which infect bacteria. By the use of the terms "bacteriophage library" or "phage library" as used herein, is meant a population of bacterial viruses comprising heterologous DNA, *i.e.*, DNA which is not naturally encoded by the bacterial virus.

The term "virus vector" includes a virus into which heterologous DNA has been inserted. The virus vector may be a bacteriophage or may be a eukaryotic virus.

By the term "target cell" as used herein, is meant a cell which expresses an antigen against which the desired antibody is sought.

By the term "panning" or "panned" as used herein, is meant the process of selecting phage which encode the desired antibody.

By the term "Fab/phage" as used herein, is meant a phage particle which expresses the Fab portion of an antibody.

By the term "scFv/phage" are used herein, is meant a phage particle which expresses the Fv portion of an antibody as a single chain.

By "excess unlabeled cells" is meant an amount of unlabeled cells which exceeds the number of labeled cells. Preferably, the ratio of labeled cells to unlabeled cells is about 1:2. More preferably, the ratio of labeled cells to unlabeled cells is greater than about 1:4. Even more preferably, the ratio of labeled cells to unlabeled cells is greater than about 1:10.

While the method as exemplified herein describes the generation of phage which encode the Fab portion of an antibody molecule, the method should not be construed to be limited solely to the generation of phage encoding Fab antibodies. Rather, phage which encode single chain antibodies (scFV/phage antibody libraries) are also included in the method. Fab molecules comprise the entire Ig light chain, that is, they comprise both the variable and constant region of the light chain, but include only the variable region and first constant region domain (CH1) of the heavy chain. Single chain antibody molecules comprise a single chain of protein comprising the Ig Fv fragment. An Ig Fv fragment includes only the variable regions of the heavy and light chains of the antibody, having no constant region contained therein. Phage libraries comprising scFV DNA may be generated following the procedures described in Marks *et a*l., 1991, *J. Mol. Biol.* **222:**581-597. Panning of phage so generated for the isolation of a desired antibody is conducted as described herein for phage libraries comprising Fab DNA.

The method should also be construed to include synthetic phage display libraries in which the heavy and light chain variable regions may be synthesized such that they include nearly all possible specificities. Therefore, antibody-displaying libraries can be "natural" or "synthetic" (Barbas, 1995, *Nature Medicine* **1:**837-839; de Kruif *et al.* 1995, *J. Mol. Biol.* **248:**97-105). Antibody-displaying libraries comprising "natural" antibodies are generated as described in the experimental example section.

Antibody-displaying libraries comprising "synthetic" antibodies are generated following the procedure described in Barbas (1995, *supra*) and the references cited therein.

The method should be further construed to include generation of phage display libraries comprising bacteriophage other than M13 as exemplified herein. Other bacteriophage, such as lambda phage, may also be useful in the method just described. Lambda phage display libraries have been generated which display peptides encoded by heterologous DNA on their surface (Sternberg *et al*., 1995, *Proc. Natl. Acad. Sci. USA* **92:**1609-1613). Moreover, it is contemplated that the method described herein may be extended to include viruses other than bacteriophage, such as eukaryotic viruses. In fact, eukaryotic viruses may be generated which encode genes suitable for delivery to a mammal and which encode and display an antibody capable of targeting a specific cell type or tissue into which the gene is to be delivered. For example, retroviral vectors have been generated which display functional antibody fragments (Russell *et al.*, 1993, *Nucl. Acids Res.* **21:**1081-1085).

The red blood cell antibodies to which antibodies may be generated include, but are not limited to, Rh antigens, including Rh(D), Rh(C), Rh(c), Rh(E), Rh(e), and other non-Rh antigens, including red blood cell antigens in the Kell, Duffy, Lutheran and Kidd blood groups.

Thus, the method for generating phage expressing antibodies is not limited solely to the isolation of DNA encoding anti-Rh(D) antibodies, but rather may be used for the isolation of DNA encoding antibodies directed against any red blood cell antigen or other cell antigen, such as, but not limited to, tumor-specific antigen, bacterial antigens, and the like. The method of the invention is also useful for typing platelets by generating phage antibodies specific for a number of clinically important platelet antigens, notably, $P1^{A1}/P1^{A2}$, Bak^{a}/Bak^{b}, Pen^{A}/Pen^{B}, and the like.

The invention is further useful for typing donor white blood cells for HLA antigens for the purposes of matching donors and recipients for potential

transplant matching in the case of both solid (for example, kidney, heart, liver, lung) and non-solid (for example, bone marrow) organ or tissue transplanting.

To detect binding of phage expressing antibody directed against one of these non-red blood cell antigens, the non-red blood cells may be agglutinated or captured following the procedures described herein for agglutination or capturing of red blood cells. Prior to agglutination or capturing, the cells may be rendered "visible" by staining or other labeling technique in order that agglutination or capturing is apparent to the naked eye or scanner.

The method is most useful for the generation of a protein which binds to an antigen-bearing moiety, where the antigen-bearing moiety is not easily purified in soluble form. Thus, antigen-bearing moieties include those which are associated with other structures, usually membranes in the cell such as cell membranes or organelle membranes.

The method is also useful for the generation of autoimmune antibodies such as those involved in autoimmune hemolytic anemia (AIHA) (Siegel *et al.*, 1994, *Structural analysis of red cell autoantibodies*, Garratty (*ed.*) *Immunobiology of Transfusion Medicine*, Dekker, New York, New York). Autoimmune antibodies that are directed against cell antigens which are cell surface membrane associated or cell organelle membrane associated may be isolated using the technology described herein. Autoimmune diseases and their associated antigens to which antibodies may be isolated include, but are not limited to the following: Myasthenia gravis (acetylcholine receptor; neurons), chronic inflammatory demyelinating polyneuropathy (myelin; neurons), autoimmune thyroid disease (thyroid stimulating hormone receptor; thyroid cells), primary biliary cirrhosis (mitochondrial autoantigens; liver mitochondria), idiopathic thrombocytopenic purpura (platelet membrane integrins; platelets), pemphigus vulgaris (epidermal antigens; epidermus), and Goodpasture's syndrome (basement membrane antigens; kidney or lung cells).

In fact, the method described herein is useful for the isolation of DNA clones encoding any antibody directed against an antigen expressed on a cell, which

cell can be labeled with a magnetic label and which cell can be obtained in sufficient quantities in an unlabeled form so as to provide an excess of unlabeled cells as required in the assay.

Further, the method is not limited to the isolation of DNA encoding antibodies but rather may also be used for the isolation of DNA encoding other peptides or proteins having specificity for cell proteins, such as, for example, but not limited to, ligands which bind cell receptor proteins, peptide hormones, and the like.

The method should also not be construed as being limited to the use of biotin as the cell-labeling agent. Other labels may be used provided their addition to a cell does not disturb the structural integrity of any surface proteins expressed thereon and provided such labels permit the addition of a paramagnetic microbead or other magnetic substance thereto. Other such labels include, but are not limited to, cell surface proteins or carbohydrates which can be directly derivitized with magnetic beads that possess activated amine, carboxyl, or thiol groups. In addition, dyes such as fluorescein or rhodamine may also be covalently attached to cells in a manner similar to biotin and magnetic beads coated with anti-dye antibodies may be attached thereto.

The invention includes proteins and DNA encoding the same which are generated using the methods described herein. To isolate DNA encoding an antibody, for example, DNA is extracted from antibody expressing phage obtained according to the methods of the invention. Such extraction techniques are well known in the art and are described, for example, in Sambrook *et al.* (*supra*).

An "isolated DNA", as used herein, refers to a DNA sequence, segment, or fragment which has been purified from the sequences which flank it in a naturally occurring state, *e.g.*, a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, *e.g.*, the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to DNA which has been substantially purified from other components which naturally accompany the DNA, *e.g.*, RNA or DNA or proteins which naturally accompany it in the cell.

The invention should also be construed to include DNAs which are substantially homologous to the DNA isolated according to the method of the invention. Preferably, DNA which is substantially homologous is about 50% homologous, more preferably about 70% homologous, even more preferably about 80% homologous and most preferably about 90% homologous to DNA obtained using the method of the invention.

"Homologous" as used herein, refers to the subunit sequence similarity between two polymeric molecules, *e.g.*, between two nucleic acid molecules, *e.g.*, two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, *e.g.*, if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, *e.g.*, if half (*e.g.*, five positions in a polymer ten subunits in length) of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, *e.g.*, 9 of 10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3'ATTGCC5' and 3'TATGCG5' share 50% homology.

To obtain a substantially pure preparation of a protein comprising, for example, an antibody, generated using the methods of the invention, the protein may be extracted from the surface of the phage on which it is expressed. The procedures for such extraction are well known to those in the art of protein purification. Alternatively, a substantially pure preparation of a protein comprising, for example, an antibody, may be obtained by cloning an isolated DNA encoding the antibody into an expression vector and expressing the protein therefrom. Protein so expressed may be obtained using ordinary protein purification procedures well known in the art.

As used herein, the term "substantially pure" describes a compound, *e.g.*, a protein or polypeptide which has been separated from components which naturally accompany it. Typically, a compound is substantially pure when at least

10%, more preferably at least 20%, more preferably at least 50%, more preferably at least 60%, more preferably at least 75%, more preferably at least 90%, and most preferably at least 99% of the total material (by volume, by wet or dry weight, or by mole percent or mole fraction) in a sample is the compound of interest. Purity can be measured by any appropriate method, *e.g.*, in the case of polypeptides by column chromatography, gel electrophoresis or HPLC analysis. A compound, *e.g.*, a protein, is also substantially purified when it is essentially free of naturally associated components or when it is separated from the native contaminants which accompany it in its natural state.

The present invention also provides for analogs of proteins or peptides obtained according to the methods of the invention. Analogs can differ from naturally occurring proteins or peptides by conservative amino acid sequence differences or by modifications which do not affect sequence, or by both.

For example, conservative amino acid changes may be made, which although they alter the primary sequence of the protein or peptide, do not normally alter its function. Conservative amino acid substitutions typically include substitutions within the following groups:

glycine, alanine;
valine, isoleucine, leucine;
aspartic acid, glutamic acid;
asparagine, glutamine;
serine, threonine;
lysine, arginine;
phenylalanine, tyrosine.

Modifications (which do not normally alter primary sequence) include *in vivo*, or *in vitro* chemical derivatization of polypeptides, *e.g.*, acetylation, or carboxylation. Also included are modifications of glycosylation, *e.g.*, those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps; *e.g.*, by exposing the polypeptide to enzymes which affect glycosylation, *e.g.*, mammalian glycosylating or deglycosylating enzymes. Also

embraced are sequences which have phosphorylated amino acid residues, *e.g.*, phosphotyrosine, phosphoserine, or phosphothreonine.

Also included are polypeptides which have been modified using ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties. Analogs of such polypeptides include those containing residues other than naturally occurring L-amino acids, *e.g.*, D-amino acids or non-naturally occurring synthetic amino acids. The peptides of the invention are not limited to products of any of the specific exemplary processes listed herein.

In addition to substantially full length polypeptides, the present invention provides for active fragments of the polypeptides. A specific polypeptide is considered to be active if it binds to an antigen-bearing moiety, for example, if a fragment of an antibody binds to its corresponding antigen in the same manner as the full length protein.

As used herein, the term "fragment," as applied to a polypeptide, will ordinarily be at least about fifty contiguous amino acids, typically at least about one hundred contiguous amino acids, more typically at least about two hundred continuous amino acids and usually at least about three hundred contiguous amino acids in length.

Typing of Red Blood Cells Using a Virus-Expressing Antibody

The invention relates to a means of typing cells with respect to the antigens which are expressed thereon.

The phage-expressing protein which is generated using the methods described herein is useful for typing red blood cells. There is a paucity of assays for the rapid testing of red blood cells, in particular, for the typing of red blood cells with respect to Rh antigen expression. Both conventional (standard Coomb's indirect assay) and non-conventional assays (Micro Typing System, Inc., described in U.S. Patent No. 5,338,689 and the protein A based assay described in WO 9531731 A) rely on the use of antibody in the assay which is expressed in eukaryotic cells, either as a monoclonal or as a polyclonal antibody.

According to the invention there are described antibodies which are expressed on the surface of a virus, preferably a bacteriophage, which may be used to rapidly type red blood cells with respect to antigens expressed thereon, preferably, Rh antigen, that is, this method of the invention involves the use of phage-generated antibodies to type blood.

The cell typing method of the invention is not limited to the use of a particular panning method for the isolation of antibody-expressing bacteriophage described herein (*i.e.*, the use of magnetic beads to pan phage), but rather, is also applicable to antibody-expressing phage which are obtained using other known phage panning methods, including, but not limited to, the fluorescent panning method described in De Kruif et al., (*supra*), and is also applicable to phage which are obtained using as yet unknown phage panning methods which may become available in the future. Thus, it is contemplated that the red blood cell typing method of the invention may be used with any bacteriophage which express antibody irrespective of the manner by which the bacteriophage are obtained.

The cell typing method of the invention is not even limited to phage display libraries which require panning for screening. Rather, a monoclonal antibody system may be converted to a phage system as follows, the antibody produced by which would not require panning. This type of system is described in Siegel *et al.* (1994, *Blood* **83**:2334-2344). Briefly, monoclonal antibody producing hybridoma cell mRNA is converted to cDNA. This DNA is amplified many times using PCR technology to generate a multiple copy library which is packaged into the bacteriophage system described herein. A library so generated does not require panning *per se;* rather, a few colonies are picked and are screened for the production of the appropriate antibody. Currently, many blood banks use mouse monoclonal antibodies directed against A and B ABO blood groups for typing of blood. Since it is ultimately cheaper to generate and use phage-displayed antibodies which may be propagated in bacteria than it is to generate and use monoclonal antibodies produced in

cells in culture, the methods of the invention provide significant advantages over currently used methods.

The method of the invention relates to the detection of an antigen on a red blood cell and comprises incubating a mixture of red blood cells, a bacteriophage having an antibody expressed thereby on the surface of the bacteriophage, wherein the antibody is specific for a red blood cell antigen, and an anti-bacteriophage antibody, and determining whether the red blood cells in the mixture have bound the phage, wherein binding of the red blood cells to the phage is an indication that the red blood cells contain an antigen which binds to the antibody expressed by the bacteriophage.

Detection of red blood cell binding to phage may be accomplished in an agglutination assay. Agglutination may be detected using conventional agglutination assays wherein red blood cells are either centrifuged or are allowed to settle to the bottom of a tube or a well and the formation of agglutinates is assessed by examining the settled cells in a concave mirror. Alternatively, agglutination may be assessed using Micro Typing System cards wherein anti-phage antibody is used in place of Coomb's reagent. Several available micro typing systems are available commercially which may be adapted for use in the present invention.

In a card typing assay, for example, but not limited to the Micro System Typing assay, a mixture of red blood cells and antibody-expressing phage is incubated and applied to a microtube containing inert particles and anti-phage antibody. The anti-phage antibody will bind only to those red blood cells which have antibody-expressing phage bound thereon, which red blood cells therefore themselves express an antigen to which the antibody expressed by the phage binds. The microtube is centrifuged in a controlled manner, wherein strong agglutination (cross linking of cells/phage-displayed antibody and the anti-phage antibody in the tube) causes essentially no movement of the red blood cell/phage-displayed antibody/anti-phage antibody agglutinate through the tube. If no agglutination has occurred then the red blood cells will form a visible pellet at the bottom of the microtube. If a weak

agglutination reaction has occurred, then some dispersion of red blood cells throughout the tube will be evident.

It is not entirely necessary that the microtube be centrifuged. Sedimentation of the components of the assay may be accomplished by allowing the vessel to stand and take advantage of the force of gravity. However, it is more advantageous to centrifuge the tube.

The microtube used to detect cell agglutination is a transparent microtube having an upper and a lower portion, the upper portion being wider than the lower portion. The microtube also has a openable top end and a closed bottom end, and is capable of withstanding a centrifugal force sufficient to pellet a population of cells.

The term "inert" as it is used to refer to particles which are present in the microtube are so termed because it is understood that they will not enter into any unspecific reactions with the particular antigens or antibodies added to the tube. Inert particles may comprise inert porous particles which are available in commerce for gas or liquid chromatography. These products are based on cross-linked polymers such as agarose, polyacrylamide, polydextran, or styrene-divinylbenzene polymers, such as Sephadex, Sepharose or Sephacryl sold by Pharmacia AB, Uppsala, Sweden. Porous glass or silica gel particles are also suitable. The particle size is preferably 10-200 microns.

The microtubes in which the assays are conducted may be arranged in the form of a test card, such as that described in U.S. Patent No. 5,338, 689. The tubes may be affixed to a card, or may form an integral part of the card in the form of blisters contained therein.

Binding of antibody expressing phage to red blood cells may also be detected in an assay which is not an agglutination assay. This assay is referred to herein as a "capturing" assay. For example, an assay may be performed in a microtube containing inert particles, such as, but not limited to, gel beads, wherein the beads are coated with anti-phage antibody. To generate beads which are coated with anti-phage

antibody, the beads may first be coated with avidin or streptavidin. This is accomplished by chemically attaching such compounds to the beads using gels which are activated with amino-reactive groups (for example, N-hydroxy succidimidyl ester or cyanogen bromide), amino, or thiol-reactive groups (for example, epoxy-activated gels), or thiol-reactive groups (for example, thiopropyl-activated gels). Many activated gel supports are available commercially, for example, from Pierce Chemical Co. (Rockford, IL) and Pharmacia Biotech (Uppsala, Sweden). In addition, gel supports which are already chemically derivatized with avidin or streptavidin are available from many vendors.

In addition, beads which are coated with anti-phage antibody may be generated by using goat anti-sheep antibodies and anti-M13 antibodies which are made in sheep (available from 5-Prime 3-Prime). Alternatively, beads may be coated with goat anti-rabbit antibodies and anti-M13 antibodies which are made in rabbits. Similarly, beads may be coated with goat anti-mouse antibodies and mouse polyclonal or monoclonal anti-M13 antibodies.

Beads may also be coated by conjugating anti-M13 antibodies with fluorescein or other compound and conjugating the beads with anti-fluorescein antibody or other appropriate antibody, depending on the compounds conjugated to the anti-M13 antibody.

A biotinylated anti-phage antibody is added to beads so coated to effect coating of the beads with the anti-phage antibody. A mixture of red blood cells and phage-expressing antibody is applied to microtubes containing beads coated with anti-phage antibody, incubation is allowed for a period of time, and the tube is centrifuged. Red blood cells which have antibody-expressing phage bound thereon will be "captured" by the anti-phage coated beads and will therefore not sediment to the bottom of the tube, whereas red blood cells which do not have phage-expressing antibody bound thereon will not be "captured" and will sediment to the bottom of the tube.

In an preferred embodiment of the invention, the antigen on the red blood cells is the Rh(D) antigen, the antibody-expressing bacteriophage is M13 and the anti-phage antibody is anti-M13 antibody.

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

A method is described in Figure 1 for the isolation of filamentous phage-displayed human monoclonal antibodies specific for unpurifiable cell surface expressed molecules. To optimize the capture of antigen-specific phage and minimize the binding of irrelevant phage antibodies, a simultaneous positive and negative selection strategy was employed. Cells bearing the antigen of interest are pre-coated with magnetic beads and are diluted into an excess of unmodified antigen-negative cells. Following incubation of the cell admixture with a Fab/phage library, the antigen positive cell population is retrieved using magnetically-activated cell sorting, and antigen-specific Fab/phage are eluted and propagated in bacterial culture. When this protocol was used with magnetically-labeled (Rh(D)-positive and excess unlabeled Rh(D)-negative human red blood cells and a Fab/phage library constructed from human peripheral blood lymphocytes, dozens of unique, clinically useful $_{\gamma 1}\kappa$ and $_{\gamma 1}\lambda$ anti-Rh(D) antibodies were isolated from a single alloimmunized individual.

The cell-surface selection method of the present invention is readily adaptable for use in other systems, such as for the identification of putative tumor-specific antigens, and provides a rapid (less than one month), high yield approach for isolating self-replicative antibody reagents directed at novel or conformationally-dependent cell-surface epitopes.

The experimental examples described herein provide procedures and results for the isolation and production of anti-Rh(D) red blood cell antibodies using

Fab/phage display. These examples also provide procedures for agglutination of red blood cells using anti-phage antibodies.

Creation of Fab/phage display libraries

Separate $_{\gamma 1}\kappa$ and $_{\gamma 1}\lambda$ phage libraries were constructed from 2 x 10^7 mononuclear cells derived from the peripheral blood from an Rh(D)-negative individual previously hyperimmunized with Rh(D)-positive red blood cells. The phagemid vector pComb3 (Barbas, 1991, *Proc. Natl. Acad. Sci. USA* **88:**7978-7982) was used to create the libraries utilizing previously published methods (Barbas *et al.*, 1991, Combinatorial immunoglobulin libraries on the surface of phage (Phabs): Rapid selection of antigen-specific Fabs. Methods: A Companion to Methods in Enzymology **2:**119-124; Siegel *et a*l., 1994, *Blood* **83:**2334-2344).

Briefly, cDNA was prepared from the mRNA of the donor cells and heavy chain and light chain immunoglobulin (Ig) cDNA segments were amplified using the polymerase chain reaction (PCR) and the battery of human Ig primers described by Kang *et al.* (1991, "Combinatorial Immunoglobulin Libraries on the Surface of Phage (Phabs): Rapid Selection of Antigen-Specific Fabs. Methods: A Companion to Methods" in *Enzymology* 2:111-118) supplemented by those of Silverman *et al.* (1995, *J. Clin. Invest.* **96:**417-426). The heavy and light chain PCR products were cloned into pComb3 and electroporated into *E. coli.* Upon co-infection with VCSM13 helper phage (Stratagene, La Jolla, CA), Ig DNA was packaged into filamentous phage particles which express human Fab molecules fused to the gene III bacteriophage coat protein.

Panning Fab phage display libraries for anti-Rh(D) clones

Rh(D)-positive red blood cells were cell-surfaced biotinylated by incubating cells at a hematocrit of 10% with 500 µg/ml sulfo-NHS-LC-biotin (Pierce Chemical, Rockford, IL) for 40 minutes at room temperature (RT). Following 5 washes with phosphate-buffered saline (PBS), 8 x 10^6 biotinylated Rh(D)-positive red blood cells were incubated with 10 µl of streptavidin-coated paramagnetic microbeads (MACS Streptavidin Microbeads, Mitenyi Biotec, Sunnyvale, CA) for 1 hour at RT in

a total volume of 100 μl PBS. Unreacted beads were removed by washing and then the magnetic bead-coated, Rh(D)-positive red blood cells were mixed with a 10-fold excess (8×10^7) of the Rh(D)-negative (unmodified) red blood cells and $\sim 3 \times 10^{11}$ colony-forming units (cfu) of either the $_{\gamma1}\kappa$ and $_{\gamma1}\lambda$ Fab/phage libraries (prepared as described above) in a final volume of 40 μl PBS containing 2% non-fat dry milk (MPBS, Carnation, Nestle Food Products, Glendale, CA).

Following a 2 hour incubation at 37°C, the red blood cell/phage suspension was loaded at a flow rate of 10 μl/minute onto a MiniMACS magnetic type MS column (Mitenyi Biotec, Sunnyvale, CA) that was pre-equilibrated with 2% MPBS. This loading step was performed <u>without</u> a magnetic field around the column so as to prevent magnetic bead-coated red blood cells from instantly adhering to the very top of the column, clogging it, and causing the trapping of Rh(D)negative unbiotinylated red blood cells. Loading the red blood cell/phage incubation mixture in the absence of a magnetic field causes the antigen-negative and antigen-positive red blood cells to distribute evenly throughout the column without running off since the excluded volume of the column is slightly greater than 40 μl. Once loaded, the column was placed in a magnetic field (MiniMACS magnetic separation unit, Mitenyi Biotec, Sunnyvale, CA) for 2 minutes to allow the Rh(D)-positive red blood cells to adhere, and a series of 500 μl washes were performed with ice-cold MPBS followed by a final wash with PBS. A total of 3 washes were performed for the first 2 rounds of panning and a total of 6 washes were performed for all subsequent pannings. For each panning, the first wash was carried out at a flow rate of 10 μl/minute during which time the bulk of Rh(D)-negative red blood cells washed off the column. All subsequent washes were performed at 200 μl/minute. Following the last wash, the column was removed from the magnetic field and the bead-coated/phage-coated Rh(D)-positive red blood cells were flushed off the column with 500 μl PBS using the plunger from a 5 cc syringe (Becton-Dickinson, Franklin Lakes, NJ).

The red blood cells were immediately centrifuged for 5 seconds at 13,000 x g and were then resuspended in 200 μl of 76 mM citrate, pH 2.4, to denature

the Rh(D) antigen and elute bound phage. Following a 10 minute incubation period at RT with intermittent vortexing, the phage eluate and cellular debris were neutralized with 18 µl 2 M Tris base and were added to 10 ml of O.D.=1.0 XL1-Blue strain of *E. coli* (Stratagene, La Jolla, CA) grown in super broth (SB) (Barbas *et al.*, 1991, *supra*) supplemented with 10 µg/ml tetracycline. After incubation for 15 minutes at RT, during which time the phage library enriched for Rh(D) binders was allowed to infect the bacterial culture, 10 ml of pre-warmed, 37°C SB containing 40 µg/ml carbenicillin/10 µg/ml tetracycline was added to give final antibiotic concentrations of 20 µg/ml and 10 µg/ml, respectively. A small aliquot of culture (~100 µl) was immediately removed and titered on LB/carbenicillin plates to determine the number of phage contained in the total eluate. The balance of the culture was shaken at 37°C for 1 hour at 300 RPM. Additional antibiotics, additional SB, and VCSM13 helper phage were subsequently added and the culture was grown overnight at 30°C as described (Siegel *et al.*, 1994, *supra*).

Phagemid particles were purified from the culture supernatant by polyethylene glycol 8000 (PEG) precipitation (Barbas *et al.*, 1991, *supra*), resuspended in 1% bovine serum albumin (BSA)/PBS, and dialyzed overnight to remove residual PEG that may lyse red blood cells during subsequent rounds of panning. Thus, the resultant phage preparation serves as the input for the next round of panning. The $\gamma_1\kappa$ and $\gamma_1\lambda$ phage libraries were panned separately to prevent any bias in light chain isotype replication possibly introduced by bacterial amplification.

Screening polyclonal Fab/phage libraries and individual phage colonies for anti-Rh(D) reactivity

The specificity of Fab/phage for the Rh(D) antigen was assessed using anti-M13 antibody as a bridging antibody to induce agglutination between red blood cells that have bound anti-Rh(D) Fab/phage. One hundred µl aliquots of polyclonal Fab/phage from rounds of panning, or monoclonal Fab/phage derived from individual Fab/phage eluate clones, were incubated with 50 µl of a 3% suspension of red blood cells of defined phenotype (*i.e.*, Rh(D)-negative or -positive).

Following 1 hour incubation at 37°C, the red blood cells were washed 3 times with 2 ml cold PBS to remove unbound Fab/phage. The resultant red blood cell pellets were resuspended in 100 µl of a 10 µg/ml solution of sheep anti-M13 antibody (5-Prime 3-Prime, Boulder, CO) and transferred to the round-bottomed wells of a 96-well microtiter plate. Plates were left undisturbed (~2 hours) and were then read. Wells having a negative reaction exhibit sharp ~2 mm diameter red blood cell spots whereas in wells having positive reactions, *i.e.*, agglutination, the red blood cells in agglutinated wells form a thin carpet coating the entire floor of the well.

For hemagglutination assays utilizing minicolumn gel cards (ID-Micro-Typing System, Ortho Diagnostics, Raritan, NJ) (Lapierre et al., 1990, Transfusion 30:109-113), 25 µl of Fab/phage clones were mixed with 50 µl aliquots of red blood cells (0.8% suspensions in Micro Typing System buffer, Ortho Diagnostics). The mixtures were placed in the reservoirs above the minicolumns which contain dextran-acrylamide beads previously suspended in 100 µl/ml anti-M13 antibody. After incubation at 37°C, the gel cards were centrifuged at 70 x g for 10 minutes and were read.

Miscellaneous methods

Preparation of fluorescently-labeled red blood cells for flow cytometry was performed as described herein and samples were analyzed using a FACScan microfluorimeter equipped with Lysis II (Ver 1.1) software (Becton-Dickinson, Mountain View, CA). Plasmid DNA was prepared from bacterial clones (Qiawell Plus, Qiagen, Chatsworth, CA). Double-stranded DNA was sequenced using light chain or heavy chain Ig constant region reverse primers or unique pComb3 vector primers that anneal 5-prime to the respective Ig chain (Barbas *et al.*, 1991, *supra*; Roben *et al.*, 1995, J. Immunol. 154:6437-6445) and automated fluorescence sequencing (Applied Biosystems, Foster City, CA). Sequences were analyzed using MacVector Version 5.0 sequencing software (Oxford Molecular Group, Oxford, UK) and the Tomlinson database of Ig germline genes (Tomlinson *et al.*, 1996, *V Base Sequence Directory*. MRC Center for Protein Engineering, Cambridge, UK).

Experimental design for cell incubation and separation protocols

The experimental conditions described above for panning Fab/phage libraries for anti-red blood cell-reactive phage were determined after performing a series of initial studies aimed at optimizing the cell separation process and ultimate yield of antigen-specific Fab/phage. The main parameters investigated included:

<u>Biotinylation</u> Conditions were sought that would biotinylate the red blood cell surface in a manner such that a sufficient number of streptavidin-coated magnetic beads would bind to the cells causing the red blood cells to be retained by a magnetic column. In this case, over-biotinylation that might destroy the antigenicity of the Rh(D) antigen or might make the cells non-specifically absorb antibody is to be avoided. To address this issue, Rh(D)-positive/Kell-negative red blood cells (Kell being a red blood cell antigen; (Walker, ed. 1993, *Technical Manual, 11th Edition, Bethesda: American Association of Blood Banks*) were incubated with a range of sulfo-NHS-LC-biotin concentrations and the degree of biotinylation was assessed by flow cytometry utilizing fluorescein-conjugated streptavidin.

To assess the degree of cell-surface biotinylation, 5 μl aliquots of 3% suspensions of Rh(D)-positive/Kell-negative red blood cells biotinylated at varying biotin reagent concentrations were incubated with 200 μl of a 1/100 dilution of FITC-streptavidin (Jackson ImmunoResearch, Bar Harbor, Maine) for 30 min at 4°C (Figure 2). The mixture was washed with phosphate buffered saline (PBS) and analyzed by flow microfluorimetry (–□–). Aliquots of cells were also analyzed for retention of Rh(D)-antigenicity (–Δ–) (*i.e.*, specific staining) or for lack of non-specific staining (–O–) by incubating the cells with 100 μl of either anti-Rh(D) or anti-Kell typing sera, respectively, washing the cells and then staining them with a 1/100 dilution of FITC-goat anti-human IgG (Jackson ImmunoResearch).

A linear, non-saturating response was observed (Figure 2). Retention of Rh(D) antigenicity was assessed using anti-Rh(D) typing serum and was found to be unaffected by the derivatization of cell-surface proteins with biotin at all biotin

concentrations tested (Figure 2). Furthermore, the Kell-negative red blood cells did not non-specifically adsorb anti-Kell antibodies.

Each biotinylated red blood cell sample was then incubated with an excess of streptavidin-coated magnetic microbeads and applied to a magnetic separation column. It was determined that as many as 10^8 red blood cells could be retained by the column for red blood cell samples biotinylated with greater than or equal to 500 μg/ml biotin reagent. Since the actual red blood cell/phage panning experiments were designed to use only ~10^7 Rh(D)-positive cells (see below), red blood cell biotinylation at 500 μg/ml was determined to be sufficient.

<u>Concentration of Rh(D)-positive and Rh(D)-negative RBCs in incubation mixture</u>

Prior to performing Fab/phage panning experiments, the ability of the magnetically-activated cell separation technique to separate Rh(D)-positive and Rh(D)-negative cells was assessed using anti-Rh(D) typing serum and flow cytometry (Figure 3). Streptavidin-microbead coated, biotinylated Rh(D)-positive red blood cells (8 x 10^6 cells) were mixed with a 10-fold excess of Rh(D)-negative uncoated red blood cells (8 x 10^7 cells) in a 40 μl volume of PBS containing 2% non-fat dry milk (MPBS) and the mixture was applied to a MiniMACS column. The column was washed and the bound cells were eluted as described herein. Aliquots of red blood cells contained in the original admixture (panel a), the column wash (panel b), and the column eluate (panel c) were stained with anti-Rh(D) typing serum and FITC-goat anti-human IgG as described in Figure 2. The flow cytograms show that although ~90% of the cells in the column load were Rh(D)-negative (panel a), nearly all of them washed off of the column (panel b), yielding a column eluate that was almost entirely Rh(D)-positive cells (panel c). Since only ~6% of the final eluate comprise Rh(D)-negative cells (panel c), and Rh(D)-negative cells were initially present in a 10-fold excess to Rh(D)-positive cells, only ~0.6% of the initial antigen-negative immunosorbant cells contaminated the final antigen-positive preparation. This efficiency of the cell separation was deemed adequate for subsequent panning experiments with Fab/phage.

In the above-described experiment, to avoid clogging the magnetic separation column, it was necessary to load the column in the absence of a magnetic field. This necessitated a reaction volume of less than or equal to 40 μl so that none of the material would run off the column. On theoretical grounds (Kretzschmar *et al.*, 1995, Anal. Biochem. 224:413-419), one can calculate the appropriate concentration of cells required in a 40 μl volume to capture greater than 50% of Fab/phage specific for a given cell surface antigen. Such a calculation is a function of the number of antigen sites per cell and the dissociation constant (K_D) of the bound Fab/phage. Using a value of ~100,000 Rh(D) antigen sites per red blood cell (phenotype "-D-/-D-") (Mollison *et al.*, 1993, *Blood Transfusion in Clinical Medicine*, Oxford, Blackwell Scientific Publications) and the desired Fab/phage affinity in the $K_D = 10^{-8}$ to 10^{-9} M range, then 8×10^6 Rh(D)-positive red blood cells in a 40 μl reaction volume would be required. Given this number of Rh(D)-positive cells, a 10-fold excess of Rh(D)-negative red blood cells was found to be the maximum amount of antigen-negative cells that could be effectively separated from antigen-positive red blood cells by the magnetic column (Figure 3).

Construction and panning of Fab/phage libraries

$_{\gamma 1}\kappa$ and $_{\gamma 1}\lambda$ phage libraries were prepared as described herein and were found to contain 7×10^7 and 3×10^8 independent transformants, respectively. Table 1 tabulates the panning results for the libraries.

A red blood cell agglutination assay utilizing anti-M13 secondary antibody as bridging antibody was used to detect anti-Rh(D) Fab/phage activity in the panned polyclonal libraries and the individual randomly-picked Fab/phage clones (Figure 4). The results shown are a representative example of the assay depicting negative reactivity to Rh(D)-negative red blood cells and strongly positive reactivity to Rh(D)-positive red blood cells for the $_{\gamma 1}\kappa$ library (panning #2) out to a dilution of 1/2048.

In the case of the $_{\gamma 1}\kappa$ library, significant enrichment for binding phage appears to occur after only one round of panning, whereas significant enrichment for

the $_{\gamma 1}\lambda$ library occurs during the second round. This is reflected by both the sharp increase in the percent of phage bound during a given round of panning as well as the ability of the polyclonal $_{\gamma 1}\kappa$ and $_{\gamma 1}\lambda$ Fab/phage libraries to agglutinate Rh(D)-positive red blood cells after 1 and 2 rounds of panning, respectively (Table 1, Figure 4).

Monoclonal Fab/phage were prepared from randomly-picked individual bacterial colonies obtained during each round of panning. It was apparent that by the third round of panning, all clones have anti-Rh(D) specificity (Table 1). To confirm that these Fab/phage have anti-Rh(D) specificity and are not binding to other unrelated antigens that may coincidentally be present on the particular Rh(D)-positive red blood cell and absent on the particular Rh(D)-negative red blood cell used in the agglutination assays, clones were screened against a panel of 11 Rh(D)-negative and-positive red blood cells of varying blood group specificities to verify their anti-Rh(D) specificity (Walker, 1993, *supra*).

Clonal analysis at the genetic level

To investigate the genetic diversity among the randomly picked anti-Rh(D) clones, plasmid DNA was prepared from each of the clones and the corresponding heavy and light chain Ig nucleotide sequences were identified. In Table 2 there is listed a number of attributes for each clone including the name of the most closely-related germline heavy or light chain Ig gene. More detailed analysis at the nucleotide level revealed that among all of the anti-Rh(D) binding clones, there were a large number of unique heavy and light chain DNA sequences (Table 3). Because of the random shuffling of heavy and light chain gene segments which occurs during the creation of a Fab/phage display library (Barbas *et al.*, 1991, *supra*), it is evident that these heavy chains and light chains combined to form nearly 50 different anti-Rh(D) antibodies.

A detailed multiple alignment analysis of the predicted amino acid sequences revealed a total of twenty-five unique heavy chain, eighteen unique kappa light chain and twenty-three unique lambda light chain proteins. Due to the combinatorial effect during library construction, these heavy and light chain gene

segments paired to produce fifty unique Fab antibodies ($20_{\gamma 1 \kappa}$ and $30_{\gamma 1 \lambda}$). Of interest, all twenty five unique heavy chains and nearly all of the eighteen unique kappa light chains were derived from only 5 V_HIII or four VκI germline genes, respectively, while the lambda light chains were derived from a more diverse set of germline genes. Analysis of heavy and light chain nucleotide sequences from over sixty negative clones from the unpanned libraries were performed to verify the heterogeneity in variable region family representation before selection. Clones representing V_H families I (13%), III (36%), IV (31%), V(15%) and VI (5%); Vκ families I (43%), II (14%), III (29%) and IV (14%); and Vγ families I (48%), II (4%), III (9%), IV (4%), V (9%), VI (17%) and VII (9%) were present.

Clonal analysis at the protein level

To investigate the diversity in fine specificity (Rh(D) antigen epitope specificity) among the anti-Rh(D) clones, agglutination experiments were performed with selected clones and with sets of rare Rh(D)-positive red blood cells which were obtained from individuals whose red blood cells produce Rh(D) antigen lacking certain epitopes. Examining the pattern of agglutination of a particular anti-Rh(D) antibody with such sets of mutant red blood cells enables the identification of the specific epitope on Rh(D) to which the antibody is directed (Mollison *et al.*, 1993, *supra*). A representative example of such an experiment is shown in Figure 5 and the Rh(D) epitopes for selected anti-Rh(D) Fab/phage clones are tabulated in Table 2.

Agglutination experiments were performed with anti-Rh(D)-negative red blood cells (rr), Rh(D)-positive red blood cells (R_2R_2), and "partial" Rh(D)-positive red blood cells (mosaics IIIa, IVa, Va, VI, VII). The results shown are a representative example of the assay for 5 randomly-picked anti-Rh(D) Fab/phage clones (Figure 5).

TABLE 1a. $_{\gamma 1}\kappa$FAB/PHAGE LIBRARY PANNING RESULTS						
PANNING[1]	φINPUT (CFUs)[2]	φOUTPUT (CFUs)[3]	%BOUND[4] (x 10^{-4})	ENRICHMENT[5]	AGGLUT TITER[6]	BINDERS/ TOTAL(%)[7]
0					0	0/16 (0)
1	2.94×10^{11}	6.04×10^{5}	2.1		1/16	0/16 (0)
2	2.15×10^{11}	1.68×10^{7}	78.3	38.0x	1/2048	15/15 (100)
3	1.72×10^{11}	1.44×10^{8}	840.0	10.7x	1/2048	12/12 (100)

TABLE 1b. $_{\gamma 1}\lambda$FAB/PHAGE LIBRARY PANNING RESULTS						
PANNING[1]	φINPUT (CFUs)[2]	φOUTPUT (CFUs)[3]	%BOUND[4] (x 10^{-4})	ENRICHMENT[5]	AGGLUT TITER[6]	BINDERS/ TOTAL(%)[7]
0					0	0/16 (0)
1	2.28×10^{11}	3.48×10^{5}	1.5		0	
2	5.51×10^{11}	1.34×10^{6}	2.4	1.6x	1/128	32/36 (89)
3	3.93×10^{11}	3.86×10^{8}	980.0	404.0x	1/512	24/24 (100)
4	2.87×10^{11}	3.08×10^{8}	1100.0	1.1x	1/1024	

1 panning round, where "0" represents the initial, unpanned Fab/phage library

2 number of colony-forming units (CFUs) of phage (φ) incubated with Rh(D)-positive/-negative RBC admixture

3 total number of CFUs of φ contained in eluate

4 (φ output/φinput) x 100

5 fold increase in % bound from compared to previous round of panning

6 agglutination titer; see text and Figure 4

7 number of Rh(D)-binding Fab/phage clones per total number of clones screened from panning round; see Table 2 for details

TABLE 2a. ANALYSIS OF $_{\gamma 1}\kappa$FAB/PHAGE CLONES						
CLONE[1]	AGGLU[2]	VH FAM[3]	VH GENE[4]	Vκ FAM[5]	Vκ GENE[6]	D EPITOPE[7]
KPO-1	neg	3	DP-47/V3-23	4	DPK24/VklVKlobeck	
KPO-2	neg	3	DP-31/V3-9P	3	DPK22/A27	
KPO-3	neg	3	DP-58/hv3d1EG	4	DPK24/VklVKlobeck	
KPO-4	neg	4	3d279d+	--	no light chain	
KPO-5	neg	3	DP-29/12-2	1	LFVK431	
KPO-6	neg	4	DP-79/4d154	1	DPK9/012	
KPO-7	neg	3	V3-48/hv3d1	4	DPK24/VklVKlobeck	
KPO-8	neg	4	DP-70/4d68	2	DPK18/A17	
KPO-9	neg	1	DP-14/V1-18	1	DPK9/012	
KPO-10	neg	4	DP-70/4d68	1	DPK9/012	
KPO-11	neg	5	DP-73/V5-51	1	DPK9/012	
KPO-12	neg	3	DP-54/V3-7	2	DPK18/A17	
KPO-13	neg	3	V3-48/hv3d1	1	Vb'	
KPO-14	neg	6	DP-74/VH-VI	1	DPK6/Vb"	
KPO-15	neg	3	DP-46/3d216	3	Vg/38K	
KPO-16	neg	6	DP-74/VH-VI	1	DPK9/012	
KP1-1	neg	4	V71-4+	3	DPK22/A27	
KP1-2	neg	4	3d279d+	1	DPK8/Vd+	
KP1-3	neg	1	4M28	1	DPK9/012	
KP1-4	neg	4	DP-79/4d154	3	Vg/38K	
KP1-5	neg	3	DP-38/9-1	3	DPK22/A27	
KP1-6	neg	4	DP-70/4d68	1	L12a/PCRdil6-5	
KP1-7	neg	5	DP-73/V5-51	2	DPK15/A19	
KP1-8	neg	4	DP-70/4d68	3	DPK22/A27	

TABLE 2a. ANALYSIS OF $_{\gamma 1}\kappa$FAB/PHAGE CLONES

CLONE[1]	AGGLU[2]	VH FAM[3]	VH GENE[4]	Vκ FAM[5]	Vκ GENE[6]	D EPITOPE[7]
KP1-9	neg	--	no heavy chain	--	no light chain	
KP1-10	neg	--	no heavy chain	3	DPK22/A27	
KP1-11	neg	1	DP-15/V1-8+	1	DPK9/012	
KP1-12	neg	3	b28e	--	no light chain	
KP1-13	neg	3	DP-47/V3-23	4	DPK24/VklVKlobeck	
KP1-14	neg	3	DP-31/V3-9P	3	DPK21/humkv328h5	
KP1-15	neg	1	DP-7/21-2	4	DPK24/VklVKlobeck	
KP1-16	neg	5	DP-73/V51	3	DPK22/A27	
KP2-1	pos	3	DP-50/hv3019b9	1	DPK9/012	epD6/7
KP2-2	pos	3	DP-50/hv3019b9	1	DPK9/012	epD6/7
KP2-3	pos	3	DP-50/hv3019b9	1	DPK9/012	epD6/7
KP2-4	pos	3	b28m	1	DPK9/012	epD2
KP2-5	pos	3	b28m	1	DPK9/012	epD1
KP2-6	pos	3	DP-50/hv3019b9	1	DPK9/012	epD6/7
KP2-7	pos	3	DP-50/hv3019b9	1	DPK9/012	epD5
KP2-8	pos	3	DP-50/hv3019b9	1	DPK9/012	
KP2-9	pos	3	DP-50/hv3019b9	1	DPK9/012	epD2
KP2-10	pos	3	DP-50/hv3019b9	1	DPK9/012	epD2
KP2-11	pos	3	DP-50/hv3019b9	1	DPK9/012	epD2
KP2-12	pos	3	DP-50/hv3019b9	1	DPK9/012	epD1
KP2-13	pos	3	DP-50/hv3019b9	1	DPK9/012	epD6/7
KP2-14	pos	3	DP-50/hv3019b9	2	DPK15/A19	epD2

TABLE 2a. ANALYSIS OF $_{\gamma 1}\kappa$FAB/PHAGE CLONES						
CLONE[1]	AGGLU[2]	VH FAM[3]	VH GENE[4]	Vκ FAM[5]	Vκ GENE[6]	D EPITOPE[7]
KP2-15	pos	3	DP-50/hv3019b9	1	DPK9/012	epD6/7
KP3-1	pos	3	DP-50/hv3019b9	1	DPK9/012	
KP3-2	pos	3	DP-50/hv3019b9	1	DPK9/012	epD6/7
KP3-3	pos	3	DP-50/hv3019b9	3	DPK9/012	
KP3-4	pos	3	DP-49/1.9111	3	DPK9/012	epD5
KP3-5	pos	3	DP-50/hv3019b9	1	DPK9/012	
KP3-6	pos	3	DP-50/hv3019b9	1	A30/SG3+	epD6/7
KP3-9	pos	3	DP-50/hv3019b9	1	DPK8/Vd+	epD6/7
KP3-8	pos	3	DP-50/hv3019b9	3	DPK9/012	epD6/7
KP3-9	pos	3	DP-50/hv3019b9	1	DPK9/012	
KP3-1O	pos	3	DP-50/hv3019b9	1	DPK9/012	
KP3-11	pos	3	DP-50/hv3019b9	1	DPK9/012	
KP3-12	pos	3	DP-46/3d216	1	DPK9/012	

1 nomenclature: prefix "KPO" denotes "$_{\gamma 1}\kappa$Fab/phage library, panning 0", "KP1" denotes "$_{\gamma 1}\kappa$Fab/phage library, panning 1", etc.

2 agglutination negative or positive against Rh(D)-positive RBC

3 Ig heavy chain variable region gene family per Tomlinson *et al.*, *supra*

4 closest related Ig heavy chain variable region gene per Tomlinson *et al. supra*

5 Ig light chain variable region gene family per Tomlinson *et al.*, *supra*

6 closest related Ig light chain variable region gene per Tomlinson *et al.*, supra

7 Rh(D) epitope as defined by rare RBC agglutination pattern (see Figure 5 and text)

TABLE 2b. ANALYSIS OF $_{\gamma 1}\lambda$FAB/PHAGE CLONES						
CLONE[1]	AGGLU[2]	VH FAM[3]	VH GENE[4]	Vκ FAM[5]	Vκ GENE[6]	D EPITOPE[7]
LPO-1	neg	4	DP-65/3d75d	1	DPL7/IGLV1S2	
LPO-4	neg	4	DP-70/4d68	6	IGLV8A1	
LPO-3	neg	6	DP-74/VH-VI	7	DPL18/VL7.1	
LPO-4	neg	3	DP-29/12-2	1	DPL3/Iv122	
LPO-5	neg	3	DP-38/9-1	6	IGLV6S1/LV6SW-G	
LPO-6	neg	1	8M27	1	DPL3/Iv122	
LPO-7	neg	1	8M27	1	DPL2/Iv1L1	
LPO-7	neg	5	DP-58/V5-51	6	IGLV6S1/LV6SW-G	
LPO-9	neg	5	DP-73/V5-51	1	DPL7/IGLV1S2	
LPO-10	neg	3	DP-38/9-1	1	DPL2/Iv1L1	
LPO-11	neg	1	DP-31/V3-9P	3	DPL23/VLIII.1	
LPO-12	neg	--	no heavy chain	1	DPL7/IGLV1S2	
LPO-13	neg	3	DP-47/V3-23	--	no light chain	
LPO-14	neg	4	DP-71/3d197d	6	IGLV6S1/LV6SW-G	
LPO-15	neg	4	DP-70/4d68	4	IGLV8A1	
LPO-16	neg	3	DP-54/V3-7	7	DPL19	
LP2-1	pos	3	DP-50/hv3019b9	1	DPL2/Iv1L1	epD2
LP2-2	pos	3	DP-77/WHG16	1	DPL3/Iv122	
LP2-3	pos	3	DP-49/1.9111	1	DPL3/Iv122	epD1
LP2-4	neg	4	3d279d+	1	DPL2/Iv1L1	
LP2-5	pos	3	DP-49/1.9111	3	DPL16/IGLV3S1	epD5
LP2-6	pos	3	DP-50/hv3019b9	1	DPL7/IGLV1S2	epd2
LP2-7	pos	3	b28m	1	DPL7/IGLV1S2	epD2
LP2-8	pos	3	DP-49/1.9111	3	IGLV3S2=Iv318	epD1

TABLE 2b. ANALYSIS OF $_{\gamma 1}\lambda$FAB/PHAGE CLONES

CLONE[1]	AGGLU[2]	VH FAM[3]	VH GENE[4]	Vκ FAM[5]	Vκ GENE[6]	D EPITOPE[7]
LP2-9	pos	3	DP-50/hv3019b9	3	DPL16/IGLV3S1	epD2
LP2-10	pos	3	DP-77/WHG16	1	DPL3/LV122	
LP2-11	neg	1	DP-75-VI-2	1	DPL5/LV117d	
LP2-12	pos	3	DP-77/WHG16	1	DPL2/LV1L1	epD2
LP2-13	pos	3	COS-8/hv3005f3	3	IGLV8A1	
LP2-14	pos	3	DP-49/1.9111	3	DPL7/IGLV1S2	epD5
LP2-15	pos	3	DP-50/hv3019b9	3	DPL16/IGLV3S1	
LP2-16	pos	3	DP-49/1.9111	2	Iv2046	epd1
LP2-17	pos	3	DP-77/WHG16=V3-21+	1	DPL3/Iv122	epD3/9
LP2-18	pos	3	DP-49/1.9111	2	VL2.1~DPL10/Iv2066	epD1
LP2-19	pos	3	DP-50/hv3019b9	3	DPL16/IGLV3S1	epD2
LP2-20	neg	3	V3-49+	3	DPL16/IGLV3S1	
LP2-21	pos	3	DP-50/hv3019b9	7	DPL18/VL7.1	epD6/7
LP2-22	pos	3	DP-49/1.9111	1	Iv2046	
LP2-23	pos	3	DP-49/1.9111	3	DPL16/IGLV3S1	epD5
LP2-24	pos	3	DP-77/WHG16	1	DPL3/Iv122	
LP2-25	pos	3	DP-50/hv3019b9	7	DPL18/VL7.1	epD6/7
LP2-26	pos	3	DP-49/1.9111	3	DPL16/IGLV3S1	
LP2-27	neg	3	COS-6/DA-8	2	VL2.1	
LP2-28	pos	1	COS-8/hv3005f3	4	IGLV8A1	
LP2-29	pos	3	DP-49/1.9111		DPL13	
LP2-30	pos	3	DP-50/hv3019b9	3	DPL16/IGLV3S1	

TABLE 2b. ANALYSIS OF $_{\gamma 1}\lambda$FAB/PHAGE CLONES						
CLONE[1]	AGGLU[2]	VH FAM[3]	VH GENE[4]	Vκ FAM[5]	Vκ GENE[6]	D EPITOPE[7]
LP2-31	pos	3	DP-50/hv3019b9	7	DPL18/VL7.1	
LP2-32	pos	3	DP-49/1.9111	1	DPL2/Iv1L1	
LP2-33	pos	3	DP-50/hv3019b9	7	DPL18/VL7.1	
LP2-34	pos	3	DP-50/hv3019b9	7	DPL18/VL7.1	
LP2-35	pos	3	DP-50/hv3019b9	3	DPL16/IGLV3S1	
LP2-36	pos	3	DP-50/hv3019b9	3	DPL16/IGLV3S1	
LP3-1	pos	3	DP-50/hv3019b9	3	DPL16/IGLV3S1	epD2
LP3-2	pos	3	DP-49/1.9111	3	DPL16/IGLV3S1	epD1
LP3-3	pos	3	DP-49/1.9111	3	DPL16/IGLV3S1	
LP3-4	pos	3	DP-50/hv3019b9	7	DPL18/VL7.1	epD6/7
LP3-5	pos	3	DP-49/1.9111	1	DPL5/LV117d	epD5
LP3-6	pos	3	DP-49/1.9111	1	DPL5/LV117d	epD1
LP3-7	pos	3	DP-77/WHG16	1	DPL2/Iv1L1	epD5
LP3-8	pos	3	b28m	1	DPL7/IGLV1S2	epD2
LP3-9	pos	3	DP-50/hv3019b9	3	DPL16/IGLV3S1	epD2
LP3-1O	pos	3	DP-50/hv3019b9	3	DPL16/IGLV3S1	
LP3-11	pos	3	DP-50/hv3019b9	3	DPL16/IGLV3S1	epD2
LP3-12	pos	3	COS-8/hv3005f3	4	IGLV8A1	epD6/7
LP3-13	pos	3	DP-50/hv3019b9	1	DPL2/Iv1L1	epD2
LP3-14	pos	3	DP-49/1.9111	3	DPL16/IGLV3S1	
LP3-15	pos	3	DP-77/WHG16	1	DPL3/Iv122	epD1
LP3-16	pos	3	DP-49/1.9111	1	DPL2/Iv1L1	epD5
LP3-17	pos	3	DP-50/hv3019b9	3	DPL16/IGLV3S1	

TABLE 2b. ANALYSIS OF $_{\gamma 1}\lambda$FAB/PHAGE CLONES						
CLONE[1]	AGGLU[2]	VH FAM[3]	VH GENE[4]	Vκ FAM[5]	Vκ GENE[6]	D EPITOPE[7]
LP3-18	pos	3	DP-50/hv3019b9	3	DPL16/IGLV3S1	
LP3-19	pos	3	DP-50/hv3019b9	3	DPL16/IGLV3S1	epD5
LP3-20	pos	3	DP-50/hv3019b9	1	DPL2/Iv1L1	
LP3-21	pos	3	DP-49/1.9111	1	DPL3/Iv122	
LP3-22	pos	3	COS-8/hv3005f3	1	DPL2/Iv1L1	
LP3-23	pos	3	DP-49/1.9111	3	DPL16/IGLV3S1	
LP3-24	pos	3	DP-50/hv3019b9	3	DPL16/IGLV3S1	

1 nomenclature: prefix "LPO" denotes "$_{\gamma 1}\lambda$Fab/phage library, panning 0", "LP1" denotes "$_{\gamma 1}\lambda$Fab/phage library, panning 1", etc.

2 agglutination negative or positive against Rh(D)-positive RBC

3 Ig heavy chain variable region gene family per Tomlinson *et al., supra*

4 closest related Ig heavy chain variable region gene per Tomlinson *et al., supra*

5 Ig light chain variable region gene family per Tomlinson *et al.*, supra

6 closest related Ig light chain variable region gene per Tomlinson *et al., supra*

7 Rh(D) epitope as defined by rare RBC agglutination pattern (see Figure 5 and text)

TABLE 3. SUMMARY OF FAB/PHAGE CLONAL ANALYSIS	
Number of unique heavy chains	25
Number of unique κ light chains	18
Number of unique λ light chains	23
Number of $_{\gamma 1}\kappa$ antibodies	20
Number of $_{\gamma 1}\lambda$ antibodies	30
Number Rh(D) epitope specificities represented	5

Use of Fab/Phage Antibodies as Blood Bank Typing Reagents

The ability of the anti-Rh(D) Fab/phage preparations to accurately distinguish Rh(D)-negative from Rh(D)-positive red blood cells in microplate hemagglutination assays (Figures 4 and 5) provided evidence that a gel test (Lapierre et al., 1990, Transfusion 30:109-1130) used by blood banks to phenotype red blood cells using conventional antisera could be adapted for use with Fab/phage.

The gel test comprises a plastic card of approximately 5 x 7 cm, containing 6 minicolumns each filed with about 20 μl of dextran-acrylamide beads suspended in anti-human globulin (Coombs reagent). Red cells to be typed are incubated with the desired human anti-sera and are centrifuged through the gel. Red blood cells which are positive for antigens to which the antisera is directed agglutinate as they encounter the anti-human globulin and become trapped in or above the gel matrix. Unreactive red blood cells sediment through the gel particles and form a pellet at the bottom of the microtube. Because the gel test offers a number of advantages over traditional blood banking methods for red blood cell phenotyping including decreased reagent volumes, the elimination of a cell washing step and a more objective interpretation of results, many blood bank facilities have adapted this new technology. As shown in Figure 6, anti-Rh-(D) Fab/phage can be used with gel cards that are modified to contain anti-M13 antibody.

To perform the assay, Rh(D)-negative or -positive red blood cells were incubated with dilutions of anti-Rh(D) Fab/phage ($_{\gamma 1}\kappa$ library, panning #2) and were centrifuged into microcolumns containing beads suspended in anti-M13 antibody. Undiluted Fab/phage stock had a titer of 5 x 10^{12} cfu/ml similar to that in the microplate settling assay (Figure 4). Because the volume of Fab/phage used in this assay is one-fourth of that in the microplate assay, the amount of Fab/phage present in the 1/625 dilution is approximately equal to that present in the 1/2048 dilution in Figure 4. Therefore, the number of Fab/phage required to yield a positive result is essentially equivalent in both assays.

In other assays which were performed as just described, when anti-M13 antibody was eliminated from the assay, no agglutination of red blood cells was observed. In addition, anti-IgG antibody does not react with recombinant Fabs expressed on the surface of the bacteriophage. Only Rh-positive cells which were reacted with anti-Rh phage were agglutinated when anti-M13 antibody was present in the assay. It should be noted that when high concentrations of anti-M13 antibody were used, even Rh-negative cells appeared to be agglutinated. This is an artifact resulting from the cross-linking of unbound (*i.e.,* unreacted) phage which becomes crosslinked in the presence of high amounts of anti-M13 antibody and forms a semi-impenetrable mat through which not all the Rh-negative cells can traverse. In the experiments described herein, and anti-M13 concentration of about 100 μg/ml was considered to be optimal for agglutination and for the prevention of false positive results. Depending on the precise concentrations of reagents and cells used in the assay, the concentration of anti-M13 may deviate from this number.

To assess the relative sensitivity of an anti-M13 modified Micro Typing System, the columns of the Micro Typing System cards had added to them 100 μg/ml of anti-M13 antibody. Rh-negative or Rh-positive red blood cells were incubated with undiluted or with five-fold serial dilutions (1/5, 1/25, 1/125, 1/625 and 1/3125) of anti-Rh phage antibodies. The cards were centrifuged and samples were assessed for agglutination. The modified Micro Typing System card assay was capable of detecting anti-Rh agglutination at a dilution of between 1/625 and 1/3125.

Procedures for isolation of tumor-specific antibodies

Fab/phage specific for tumor cells are useful for *in vitro* diagnosis (lab assays of biopsy, fluid, or blood samples), *in vivo* labeling of tumor/metastasis (coupling of antibody to imaging probe), or for treatment of malignancy (coupling of antibodies to chemical or radioactive toxins). Tumor-specific antibodies are also useful for the identification of novel antigens or markers on tumor cells which may form the basis for anti-tumor vaccines. Further, tumor-specific antibodies useful for the generation of anti-idiotypic antibodies may also form the basis for anti-tumor vaccines.

Anti-tumor antibodies are generated essentially as described herein for the generation of anti-Rh antibodies. Tumor cells, for example, but not limited to, malignant melanoma cells, are cell-surface biotinylated, labeled with streptavidin-magnetic microbeads, and are then mixed with excess normal melanocytes. Fab/phage libraries are generated from peripheral blood lymphocytes of melanoma patients who possess therapeutically useful anti-tumor antibodies. A number of melanoma patients who have "cured" themselves apparently have done so by mounting a humoral (*i.e.*, antibody) immune response. These Fab/phage libraries are incubated with the admixture of cells. Fab/phage which are directed against epitopes specific for malignant cells will bind to the malignant cells and may then be isolated utilizing the magnetic column panning approach.

Isolation of Fab/phage that identify bacterial virulence factors

The approach described herein may be used to isolate Fab/phage capable of detecting differences between the virulent bacteria and their nonpathogenic counterparts. In this case, the virulent strain of bacteria is magnetically labeled, diluted with the non-pathogenic counterpart, and an Fab/phage library which is generated from lymphocytes obtained from individuals infected with the virulent strain is added. Fab/phage which are isolated in this manner may be useful for the identification of novel bacterial antigens against which antibacterial compounds and/or vaccines may be developed.

The disclosures of each and every patent, patent application and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.